In the Claims

Please substitute the following claims:

1 (previously amended). A vector for secretory expression of an intact MK family protein by methylotrophic yeast, said vector comprising a gene encoding a mature MK family protein ligated to a signal sequence of α1 factor from *Saccharomyces cerevisiae.*

2 (previously amended). The vector according to claim 1 comprising components (a) to (g) below:

(a) a promoter sequence of a methanol-inducible alcohol oxidase gene (AOX1) from *Pichia pastoris,*

(b) a signal sequence of α1 factor from *Saccharomyces cerevisiae,*

(c) a gene encoding a mature MK family protein, wherein said gene is ligated to (b),

(d) a transcription termination sequence of a methanol-inducible alcohol oxidase gene (AOX1) from *Pichia pastoris,*

(e) a selection marker gene functioning in *Escherichia coli* and methylotrophic yeast,

(f) a replication origin functioning in *Escherichia coli,* and

(g) 5' and 3' sequences within the AOX1 gene for the site-specific homologous recombination to a methylotrophic yeast chromosomal DNA.

3 (original). The vector according to claim 1, wherein said MK family protein is MK protein.

4 (original). The vector according to claim 1, wherein said MK family protein is PTN protein.

5 (previously amended). A transformant comprising methylotrophic yeast transformed with a vector for secretory expression of an intact MK family protein, said vector comprising a gene encoding a mature MK family protein ligated to a signal sequence of α1 factor from *Saccharomyces cerevisiae*.

6 (previously amended). The transformant according to claim 5, wherein said transformant is pPIC9DP-hMK/SMD1168, said MK family protein is MK protein, and said methylotrophic yeast is strain SMD1168.

7 (previously amended). The transformant according to claim 5, wherein said transformant is pPIC9-hPTN/GS115, said MK family protein is PTN protein, and said methylotrophic yeast is strain GS115.

8 (previously amended). A method for producing an intact MK family protein, said method comprising culturing a transformant comprising methylotrophic yeast transformed with a vector for secretory expression of an intact MK family protein, said vector comprising a gene encoding a mature MK family protein ligated to a signal sequence of α1 factor from *Saccharomyces cerevisiae* and recovering secretory expression products.

9 (previously amended). The method according to claim 8, said method comprising:

(a) culturing a transformant comprising methylotrophic yeast transformed with a vector for secretory expression of an intact MK family protein, said vector comprising a gene encoding a mature MK family protein ligated to a signal sequence of α1 factor from *Saccharomyces cerevisiae*, wherein said transformant is pPIC9DP-hMK/SMD1168, said MK family protein is MK protein, and said methylotrophic yeast is strain SMD1168,

(b) inducing the expression of MK protein under the conditions of 20°C and pH 3 after proliferation at pH 4, and

(c) recovering secretory expression products.

10 (currently amended). The transformant, according to claim 5, wherein said vector comprises

(a) a promoter sequence of a methanol-inducible alcohol oxidase gene (AOX1) from *Pichia pastoris*,

(b) a signal sequence of α1 factor ~~derived~~ from *Saccharomyces cerevisiae*,

(c) a gene encoding a mature MK family protein, wherein said gene is ligated to (b),

(d) a transcription termination sequence of a methanol-inducible alcohol oxidase gene (AOX1) from *Pichia pastoris*,

(e) a selection marker gene functioning in *Escherichia coli* and methylotrophic yeast,

(f) a replication origin functioning in *Escherichia coli*, and

(g) 5' and 3' sequences within the AOX1 gene for the site-specific homologous recombination to a methylotrophic yeast chromosomal DNA.

11 (previously added). The transformant, according to claim 5, wherein said MK family protein is MK protein.

12 (previously added). The transformant, according to claim 5, wherein said MK family protein is PTN protein.

13 (previously added). The method, according to claim 8, wherein said transformant is pPIC9DP-hMK/SMD1168, said MK family protein is MK protein, and said methylotrophic yeast is strain SMD1168.

14 (previously added). The method, according to claim 8, wherein said transformant is pPIC9-hPTN/GS115, said MK family protein is PTN protein, and said methylotrophic yeast is strain GS115.

Remarks

Claims 1-14 are pending in the subject application. By this Amendment, the applicants have amended claim 10. No new matter has been added by this amendment. Entry and consideration of the amendment presented herein is respectfully requested. Accordingly, claims 1-14 are currently before the Examiner for consideration.

The applicants have amended claim 10 to remove the term "derive" which was inadvertently not amended in the Amendment dated August 14, 2003.

Also, attached herewith is an article entitled "High Yield Protein Production from *Pichia* pastoris Yeast: A Protocol for Benchtop Fermentation" by Julia Cina, Ph.D. Dr. Cina's article is submitted in support of applicants' arguments at page 8, paragraph 3 of their Amendment dated August 14, 2003 that the pH previously considered to be optimal for growth of *Pichia* yeast was 5. (see, for example, page 6 lines 4-5 of the Cina article).

In view of the foregoing amendments and remarks above, the applicants believe that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

The applicants also invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephone interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,

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Attachment: High Yield Protein Production from *Pichia* pastoris Yeast: A Protocol for Benchtop Fermentation" by Julia Cina, Ph.D.

High Yield Protein Production from *Pichia pastoris* Yeast:

A Protocol for Benchtop Fermentation

By Julia Cino, PhD

Introduction

Over the last several decades, geneticists have learned how to manipulate DNA to identify, excise, move and place genes into a variety of organisms that are quite different genetically from the source organism. A major use for many of these recombinant organisms is to produce proteins. Since many proteins are of immense commercial value, numerous studies have focused on finding ways to produce them inexpensively, easily and in a fully functional form.

The production of a functional protein is intimately related to the cellular machinery of the organism producing the protein. *E. coli* has been the "factory" of choice for the expression of many proteins because its genome has been fully mapped and the organism is easy to handle; grows rapidly; requires an inexpensive, easy-to-prepare medium for growth; and secretes protein into the medium which facilitates recovery of the protein. However, *E. coli* is a prokaryote and lacks intracellular organelles, such as the endoplasmic reticulum and the golgi apparatus that are present in eukaryotes, which are responsible for modifications of the proteins being produced. Many eukaryotic proteins can be produced in *E. coli* but are produced in a nonfunctional, unfinished form, since glycosylation or post-translational modifications do not

occur. Therefore, researchers have recently turned to eukaryotic yeast and mammalian expression systems for protein production.

Pichia Pastoris Expression System

One such eukaryotic yeast is the methanoltrophic *Pichia pastoris*. *Pichia pastoris* has been developed to be an outstanding host for the production of foreign proteins since its alcohol oxidase promoter was isolated and cloned; its transformation was first reported in 1985 [1,2]. Compared to other eukaryotic expression systems, *Pichia* offers many advantages, because it does not have the endotoxin problem associated with bacteria nor the viral contamination problem of proteins produced in animal cell culture. Furthermore, *P. pastoris* can utilize methanol as a carbon source in the absence of glucose. The *P. pastoris* expression system uses the methanol-induced alcohol oxidase (AOX1) promoter, which controls the gene that codes for the expression of alcohol oxidase, the enzyme which catalyzes the first step in the metabolism of methanol. This promoter has been characterized and incorporated into a series of *P. pastoris* expression vectors. Since the proteins produced in *P. pastoris* are typically folded correctly and secreted into the medium, the fermentation of genetically engineered *P. pastoris* provides an excellent alternative to *E. coli* expression systems. A number of proteins have been produced using this system, including tetanus toxin fragment, Bordatella pertussis pertactin, human serum albumin and lysozyme. (3 - 7).

Minimizing Growth Limiting Factors

Another advantage of *Pichia pastoris* is a prolific growth rate. Therefore, it would seem easy enough to culture it in a shake flask. This seeming advantage, however, can pose a host of problems, including pH control, oxygen limitation, nutrient limitation and temperature fluctuation. Researchers at **New Brunswick Scientific** (Edison, NJ) found that by switching from a shaker to a fermentor, protein production in *Pichia* could be increased by over 140% (3). The fermentor enables dissolved oxygen (DO) levels to be raised, not just by increasing agitation, but by increasing air flow, by supplementing the air stream with pure oxygen, or by doing all three either in series or in parallel. Nutrient limitation can also be minimized, since fermentors can be run in fed-batch mode, where fresh media or growth limiting nutrients can be pumped into the vessel at a rate that is capable of replenishing the nutrients that are depleted. Shakers can only run in a batch mode, meaning that the growth of the cells is limited by the nutrients present in the medium at the time of inoculation. The fermentor's fed-batch mode further enables methanol flow rates to be controlled to condition the cells to the presence of the methanol, as well as provide methanol at the proper rate to allow addition of just enough methanol for protein synthesis while preventing excess methanol addition which can cause toxicity.

Researchers have found that optimum protein production in *P. pastoris* occurs at 30°C, and that all protein expression ceases at 32°C. However, high heat loads occur when *P. pastoris* is actively growing or expressing high levels of protein. In actively growing shake flask cultures, it is not uncommon for the temperature to increase 25°C if left uncontrolled. Therefore, it is

imperative that all aspects of the fermentor, including temperature controller, heat exchanger, vessel and piping, must be designed to regulate temperature for optimum protein production. In addition to the fermentor's internal controller, an external bioprocessing software (BioCommand®, **New Brunswick Scientific**) is routinely used to supervise the process, as well as to provide optimal nutrient feed rates, based on either the current status of the culture or to actuate pre-determined control scenarios.

Fermentation Protocol

Research was conducted in BioFlo® 3000 benchtop fermentors (**New Brunswick Scientific**) (Figure 1) with interchangeable, autoclavable vessels of 1.25 to 10 L working volume, as well as in a BioFlo 4500 fermentors with sterilizable-in-place vessels of 15 L and 20 L working volume, (**New Brunswick Scientific**). However, these procedures can be adapted to other size fermentors thereby making the protocols scalable. In the author's laboratories, *P. pastoris* fermentations are run as multi-stage fed-batch processes with oxygen supplementation (Table 1). Here, oxygen is supplied automatically to meet the dissolved oxygen requirements for high-density cell growth.

Method for a Typical Culture

A frozen vial of 1 ml *P. pastoris* sample was inoculated into a 1 L shake flask with 150 mL Yeast Nitrogen Base (YNB)-glycerol medium. A variety of genetically engineered *P. pastoris* strains were used, many of which are slow growing on methanol (mut^s) and engineered to produce proteins of interest. The culture was incubated at 30°C, 240 rpm, for 14 hours in an

environmental incubator shaker (**New Brunswick Scientific**). The entire 150 mL volume of inoculum was transferred to a 3.3 L fermentor vessel (total volume) containing 1.5 L of basal salts medium (see media components, Table 2) plus 4.4 mL/L trace metal solution (4). The temperature was controlled at 30°C. The dissolved oxygen was set at 30% and pH is at 5.0. Ammonium hydroxide solution (30%) was used as the base solution to adjust the pH. After 20 hours of batch culture, the optical density (OD) reaches 42. The glycerol fed-batch process was then initiated. The feeding medium consisted of 50% glycerol and 12 mL/L of trace metal solution. The feed rate was 24 mL/L/h, which was adjusted automatically based on the DO reading. DO control was maintained by the proportional integral derivitive (PID) cascade controller, which changes the speed of agitation. Pure oxygen was automatically supplied to the fermentor to keep the DO level at the setpoint after the agitation speed reached the maximum allowable setpoint. After the growth phase, a half-hour carbon-source starvation period was established before the culture was switched to the production phase.

The production phase (methanol feeding) was started after 43 hours of cell growth. The production feed medium consists of 100% methanol and 12 mL/L trace metal solution. Feeding rates were divided into three stages: 6 hr induction, 48 hr in a high-feed-rate stage and 44 hr in a low-feed-rate stage. The feeding rate of the induction stage was ramped from 1 to 10.9 mL/L/hr, which was controlled by the computer program. Feeding rates in the high and low rate stages were 15 and 2 mL/L/hr respectively. The total volume of feed was 2 L. During the fermentation, oxygen demand can be quite high and oxygen was added to the air stream automatically. (Figure 2)

pH is usually adjusted to inhibit the activities of proteinases existing in the culture broth during the production phase. Furthermore, since a host strain that is protease deficient was used, it was not necessary to change the pH level when culture was shifted from cell growth phase to production phase. It has been found that pH 5 is the optimal for cell metabolism and cell growth and that the oxygen consumption rate is higher at that pH. Using this protocol, optical densities of up to 630 can be obtained. Protein expression, of course, varies with the particular protein being expressed.

Although not without problems related to its culture, *P. pastoris* culture protocols are scalable and have become a powerful tool for the production of commercially valuable proteins.

More information on *P. pastoris* culture (expression vectors, protocols, etc.) can be obtained from **Invitrogen, Inc.**, Carlsbad, CA and from *Pichia Protocols* published by Humana Press and edited by David R. Higgins and James M. Cregg

TIME (Hrs)	STAGE	MODE	FEED SUBSTANCE*	FEED RATE (ml/L/hr)
0-20	Growth	Batch	None	N.A.
20-42.5	Growth	Fed-Batch	50% Glycerol	24**
42.5-43	Starvation	Batch	None	N.A.
43-49	Induction	Fed-Batch	100% Methanol	1-10.9***
49-97	Production	Fed-Batch	100% Methanol	15
97-141	Production	Fed-Batch	100% Methanol	2

Table 1

***All feed solutions contain 12 ml/L of a trace metals solution.**

****Feed rate adjusted based on dissolved oxygen levels via BioCommand®**

***** *Linear ramp programmed via the "Time Profile" of BioCommand®**

Figure 1: BioFlo 3000 fermentor with supervisory control system.

Figure 2: Pure oxygen supplementation profile of a *P. pastoris* culture showing the increased oxygen requirement of the culture.

Table 2:
MEDIUM COMPONENTS AND FEED SOLUTIONS

H_3PO_4 - 27 ml/L

$CaSO_4 \cdot 2\ H_2O$ - 0.9 g/L

K_2SO_4 - 18 g/L

$MgSO_4 \cdot H_2O$ - 15 g/L

KOH - 4.13 g/L
Trace Metals Solution - 4.4 ml/L
Glycerol - 40 g/L

Trace Metals Solution

Cupric sulfate $\cdot\ 5\ H_2O$ - 6.0 g/L

Sodium iodide - 0.08 g/L

Manganese sulfate $\cdot\ H_2O$ - 3.0 g/L

Sodium molybdate - 0.2 g/L
Boric acid - 0.02 g/L
Cobalt chloride - 0.5 g/L
Zinc chloride - 20 g/L

Ferrous sulfate $\cdot\ 7\ H_2O$ - 65.0 g/L

Biotin - 0.2 g/L
Sulfuric acid - 5.0 ml
Water - to 1.0 liter

Feed Solutions
Glycerol Feed Solution:
50% glycerol with 12 ml/L trace metals solution

Methanol Feed Solution:
100% glycerol with 12 ml/L trace metals solution

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